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Abstract

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Keywords: Castration, monitoring, PCV2-specific IgG-antibodies, testicular tissue fluid, vaccination status

Überprüfung der Tauglichkeit von Hodengewebeflüssigkeit kastrierter Ferkel zur Verifizierung des Impfstatus der Sau und zur Herdenüberwachung

In einem „proof of concept" wurden PCV2-spezifische IgG-Antikörper in Hodengewebe-Flüssigkeit von sieben Tage alten, kastrierten Ferkeln zur Verifizierung des Impfstatus von Muttersauen gemessen. Zwölf zufällig ausgewählte Muttersauen wurde zweimal während des letzten Trächtigkeitsdrittels ein PCV2-Impfstoff und zehn Muttersauen zur Kontrolle nur Adjuvans appliziert. PCV2-spezifische Antikörpertiter von Serum und Kolostrum der Sauen wurde mit Serum und Hodengewebeflüssigkeit ihrer kastrierten Nachkommen verglichen. Geimpfte Sauen hatten mit 29250 ELISA- Units (EU) im Serum und 65410 EU im Kolostrum signifikant höhere PCV2 spezifische IgG-Antikörpertiter verglichen mit den Serum- (980 EU) respektive den Kolostrumtitern (2630 EU) der Kontrollsauen. Ferkel von geimpften Sauen wiesen mit 9362 EU im Serum und 4022 EU in der Hodengewebeflüssigkeit ebenfalls signifikant höhere Antikörper-Konzentrationen auf als Ferkel von Adjuvans behandelten Kontrollsauen, welche mit 247 EU im Serum und 354 EU in der Hodengewebeflüssigkeit aufwiesen. Es bestand eine hohe lineare Korrelation zwischen PCV2-spezifischen IgG- Antikörper Titern im Serum und den Titern in der Hodengewebeflüssigkeit ($r_s = 0{,}9148$). Der Nachweis von PCV2-spezifischen IgG-Antikörper-Titern in der Hodengewebeflüssigkeit von fünf zufällig ausgewählten Ferkeln erlaubte eine korrekte Identifizierung des Impfstatus einer Herde mit einer Zuverlässigkeit von 98% bei den geimpften und 73% bei den nicht geimpften Sauen. Die Resultate zeigen, dass anstelle zeitaufwändiger und belastender Blutprobenentnahmen, Hodengewebe von kastrierten Ferkeln für Monitoringzwecke oder zur Feststellung des Impfstatus bei den Sauen verwendet werden könnte.

Schlüsselwörter: Hodengewebeflüssigkeit, Impfstatus, Kastration, Monitoring, PCV2-spezische IgG-Antikörper

Introduction

Infection with porcine circovirus type 2 (PCV2) is a prerequisite for development of postweaning multisystemic wasting syndrome (PMWS), nowadays termed as PCV2-systemic disease (PCV2-SD).[21] Other disease manifestations, such as subclinical infections, porcine dermatitis nephritis syndrome (PDNS), or porcine respiratory disease complex (PRDC) and reproductive disorders are also attributed to PCV2 infections.[15] They are termed in Europe as porcine circovirus diseases (PCVD)[1] and in the USA as porcine circovirus associated diseases (PCVAD)[16] of which PCV2-SD is the most common.[15,21] PCV2 occurs worldwide and subclinical infection is common.[20,22] In the UK, PCV2 subclinical infection (decreased average daily weight gain without other evident clinical signs) is causing the highest economic impact, followed by PCV2-SD.[2] High maternal antibody titers against PCV2 are protective against PCV2-SD[1] and low titers in serum of sows are correlated with increased mortality in piglets.[4] A meta-analysis identified a low colostrum intake as the most important risk factor of PCV2-SD.[23] Vaccination of pregnant sows with a PCV2 vaccine in the last third of gestation induces PCV2-specific IgG-antibodies in their serum, which leads to enrichment in the colostrum conferring protective passive immunity to piglets via colostrum transfer.[12] Moreover, vaccination of pregnant sows lowers piglet mortality, reduces incidences of PCV2-SD and antibiotic usage, and improves production parameters.[7,10,17,12] Additionally, sow vaccination seems to decrease herd infection pressure.[12] PCV2-SD commonly affects pigs of 2–4 months of age. To prevent diseases, vaccination against PCV2 is an important part of breeding farm management and fatteners are keen to know the vaccination status of purchased feeder pigs. Unfortunately, maternal-derived antibody titers of vaccinated and unvaccinated sows decrease similarly during the suckling period to a comparably low level at the end of the weaning period in the 10th week of life.[12]

Concentration of maternal antibodies in piglets depends on many factors including vaccination status of the sow and colostrum intake of the piglets. Immunoglobulin G (IgG) can be detected in serum as well as in tissues. Neonatal Fc-receptors, also known as Brambell receptors, facilitate the transport of IgG between serum and interstitial fluid by transcytosis. Convective transport through paracellular pores is another possibility for IgG extravascularization into the interstitial space. A concentration gradient across the paracellular pores causes antibodies to move from the serum to the interstitial space.[14,24]

Of note, two different vaccine strategies against PCV2 are available: active immunizations of piglets in the third week of life or vaccination of the pregnant sows, both being viable options to counteract PCVD. As a proof of concept and representative for other vaccines, we developed a simple procedure to exemplarily verify PCV2 vaccination status of breeding sows and to monitor the herds by measuring PCV2-specific IgG antibodies in testicular tissue fluid. This information provides fatteners a greater certainty that weaners from breeding farms have a higher resilience against developing disease when their mothers had previously been vaccinated. To this end, we correlated PCV2-specific IgG-antibody titers of serum and colostrum of sows with titers of sera and testicular tissue fluids of their progeny. To render the method practical under field conditions, the minimum sample size required for reliable differentiation of piglets from vaccinated and unvaccinated sows was calculated.

Materials and methods

Study design and vaccination protocol

This study was carried out according to the Swiss Animal Welfare guidelines (study number 06/07) in a farrow-to-feeder herd with 130 sows of the Large White breed. The herd was free of enzootic pneumonia, actinobacillosis, porcine respiratory and reproductive syndrome virus (PRRSV), *Brachyspira hyodysenteriae*, and the mastitis prevalence was lower than 2%. On the farm the sows were not vaccinated against PCV2, but the piglets were actively immunized with a subunit vaccine against PCV2 in the 3rd week of life. Twelve randomly selected sows were vaccinated twice, first on day 65 (± 2 days) and then between days 80 and 85 of gestation with two ml of Circovac® (Merial SA, Lyon, France), an inactivated PCV2 vaccine, which was administered deep into the neck musculature. A dose contained ≥ 3.6 $\log_{10}$ antigen units, 0.2 mg thiomersal and 500 mg paraffin oil as adjuvant. Ten sows served as controls and were inoculated with 1.4 ml sham-adjuvants using the same vaccination scheme. Cross fostering of piglets was not allowed.

Blood collection

Ten ml blood was collected from a jugular vein of the sows immediately before the first PCV2 vaccination at day 65 of gestation and repeated at day 100, three weeks after the second vaccination. Two to five ml of blood was collected during isoflurane anesthesia from male piglets immediately after castration, on day seven or at weaning, on day 26. The blood was centrifuged at 3,000 rpm for five minutes and the serum stored at -20 °C.

Colostrum collection

A total of ten ml colostrum was collected from as many sow teats as possible during parturition, before suckling

of the piglets. The colostrum was stored for 36 h at 4 °C in 12 ml glass/plastic tubes in an upright position. The fat-containing supernatant was then removed and the remaining colostrum harvested and stored at -20 °C.

Generation of testicular tissue fluid

Hundred eleven seven day-old male piglets were castrated under isoflurane anesthesia according to Swiss regulations. Both testes were collected and stored at -20 °C. Two different protocols were tested to produce sufficient testicular tissue fluid. In the first, the tissues were thawed and then subjected to two more freeze-thaw cycles at -20 °C, with 0.5 - 1 ml testes tissue fluid being collected. In the second approach, 19 individual testes were minced with a scalpel before two freeze cycles. The anti-PCV2 antibody concentrations of the minced tests were 2162 ± 668 IU compared to 2318 ± 624 IU after two freeze-thaw cycles ($p=0.18$). Nevertheless, mincing of the testes produced a tissue suspension that made pipetting of free tissue fluid difficult. Therefore, we followed the protocol without mincing the organ, instead freeze-thawing the intact testes to determine antibody concentrations.

Serological examination

PCV2-specific IgG-antibodies were measured from serum of sows and piglets, from colostrum of sows and from testicular fluid of castrated piglets using SERELISA® PCV2 Ab Mono Blocking System, Zoetis, according to the manufacturer's instructions.[8,12]

Colostral antibody titers were several times higher than corresponding serum antibody titers as described by others.[5,18] Therefore the fat-free colostrum samples were diluted with the diluent supplied in the kit at a ratio of 1:20 to prevent saturation of the ELISA. The dilution was directly used in the ELISA assay according to manufacturer's instructions. Resulting ELISA units were multiplied by a dilution factor of 20 to obtain the nominal values. Sera and testicular tissue fluids with ELISA units of 20,000 were retested at a 1:3 dilution and the nominal values calculated accordingly.

PCV2 serum concentrations determined by real-time PCR

PCV2-DNA in serum of sows was determined by quantitative PCR as described by Wiederkehr et al[25].

Statistical analysis

The program JMP® 9.0.0 (SAS Institute Inc.) was used for statistical analyses. The Shapiro Wilk test was used to determine that the data were not normally distributed. Differences between groups were analyzed using the Wilcoxon rank-sum test. Differences were considered significant at $P < 0.05$. Correlations were analyzed using Spearman's rank correlation coefficient (r_s). Receiver operating characteristic (ROC) curves were used to determine the optimum cut-off value for the antibody titer in testicular tissue fluid to differentiate between piglets from vaccinated and sham-vaccinated sows.

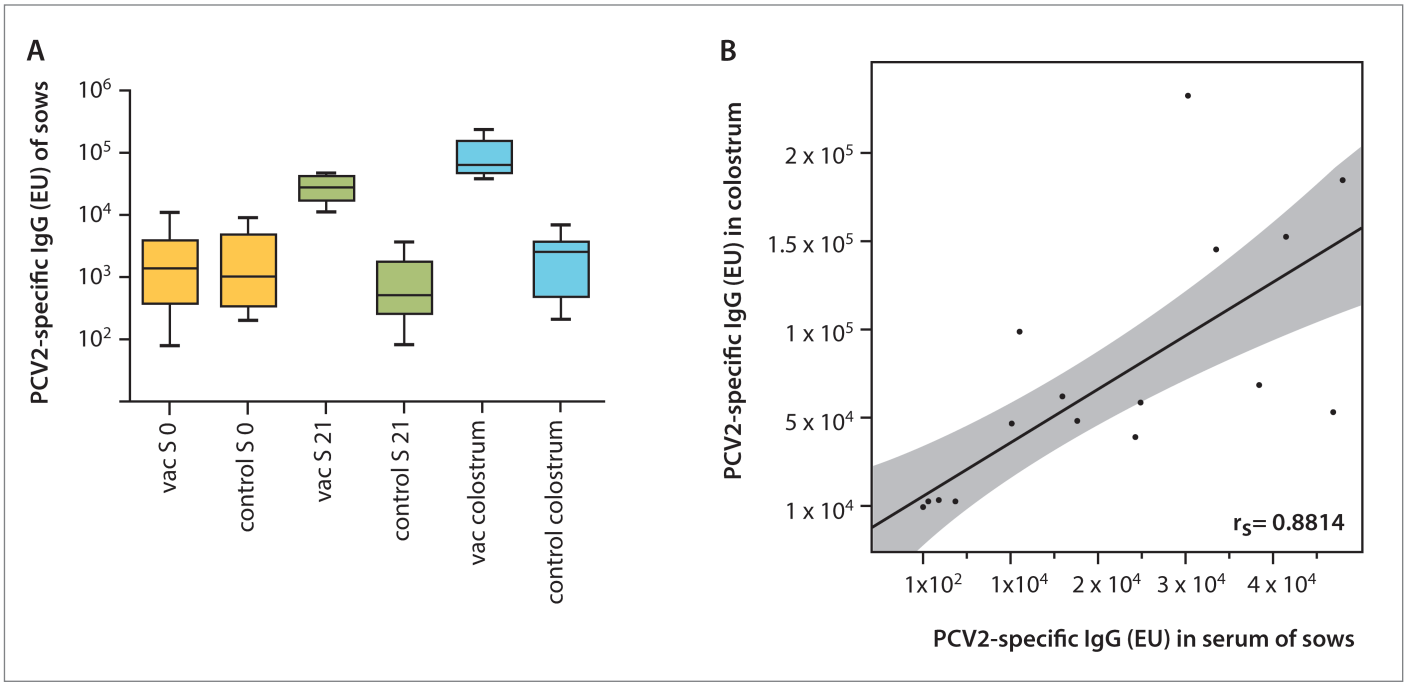

Figure 1: Comparison of PCV2-specific IgG-antibody titers in serum and colostrum of vaccinated (vac) versus control sows. **A**, Serum antibody titer in ELISA units (in EU) before vaccination (S 0), 21 days after vaccination (S 21) and colostrum. **B**, Linear correlation between PCV2-specific IgG-antibody titers (in EU) of serum and colostrum from vaccinated and control sows.

Results

PCV2 quantification and anti-PCV2 IgG-antibody titer determination in sow serum and colostrum

PCV2 was detected neither in serum of sows nor in colostrum by quantitative PCR. PCV2-specific IgG-antibody concentrations at the time of vaccination did not differ between vaccinated and adjuvant-only treated sows (P = 0.82; Fig. 1A). There was a significant increase in mean PCV2-specific antibody serum titers from 2614 EU (78 - 11379) to 29,250 EU (11,148 - 48,069 EU) upon sow vaccination between the first and second blood sampling (P < 0.001). In controls, the serum titers decreased from 2306 EU (216 - 8850 EU) to 980 EU (97 - 3720 EU) during the same time period. Colostral antibody titers varied significantly between vaccinated and control sows 65410 EU (38980 - 232920 EU) vs 2630 EU (220 - 8340 EU), (P < 0.001). The correlation coefficient (r_s) between antibody titer in serum of sows and colostrum was 0.88 (P < 0.001, Fig. 1B).

Virus quantification and PCV2-specific IgG-antibodies of serum and testicular tissue fluid of piglets

PCV2 was detected neither in serum of piglets nor in testicular tissue fluid by quantitative PCR.

58 male, seven day-old piglets from vaccinated sows were compared with 53 piglets from control sows. PCV2-antibody titers in serum and testicular tissue fluid were significantly different between piglets from vaccinated and control sows ($P < 0.001$). From the vaccinated mothers the median serum titers of the piglets were 9362 EU (774 - 56844 EU) respectively 247 EU (18 - 5584 EU) from the control mothers (Fig. 2A). The median titers in testicular tissue fluid were 4022 EU (185 - 24327 EU) from the vaccinated mothers and 354 EU (0 - 2218 EU) from the controls. There was a significant decrease in serum titers of piglets in both groups at weaning on day 26 (P < 0.05). The correlation coefficient (r_s) between the antibody titers in serum at the time of castration and the titers in testicular tissue fluid were 0.93 (P < 0.001, Fig. 2B).

PCV2-specific IgG-antibody titers from serum (Fig. 3A) and testicular tissue fluid (Fig. 3B) varied greatly among piglets of the same litter. The mean tissue fluid titers were on average 4.3 times lower than the corresponding mean serum titers. The correlation coefficient (r_s) between mean colostral antibody titers and mean serum antibody titers of piglets of the same litter were 0.92 (P < 0.001, Fig. 3C).

PCV2-specific IgG-antibodies of testicular tissue fluid predict vaccination status of the herd

For simplification of the following calculations, we assumed that all sows in a herd were either vaccinated or unvaccinated. The cut-off value of 819 EU in the testic-

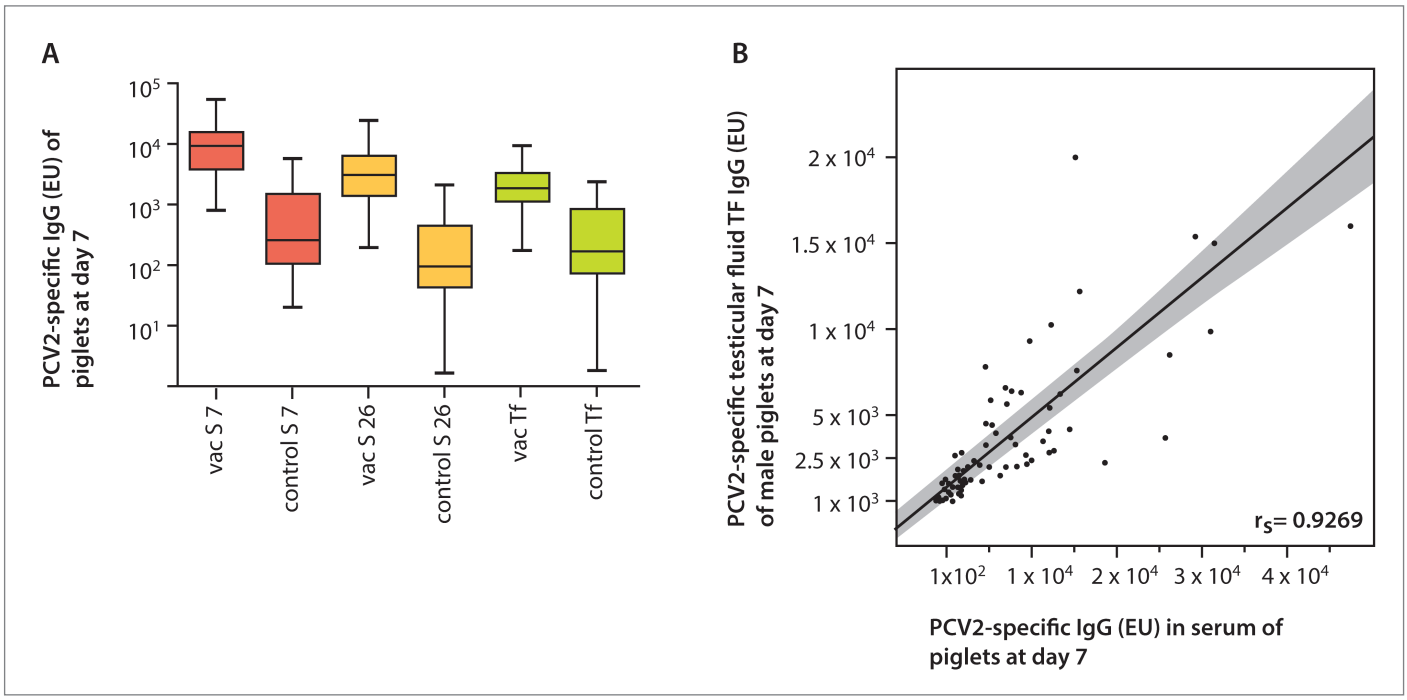

Figure 2: Comparison of PCV2-specific IgG-antibody titers from piglets serum and testicular fluid. **A**, Serum antibody in EU at castration on day 7 (S 7) and at weaning (S 26) and testicular tissue fluid (Tf) titers in EU of male piglets from vaccinated (vac) and control sows. **B**, Correlation between PCV2-specific IgG-antibody titers (in EU) from serum and testicular tissue fluid at castration.

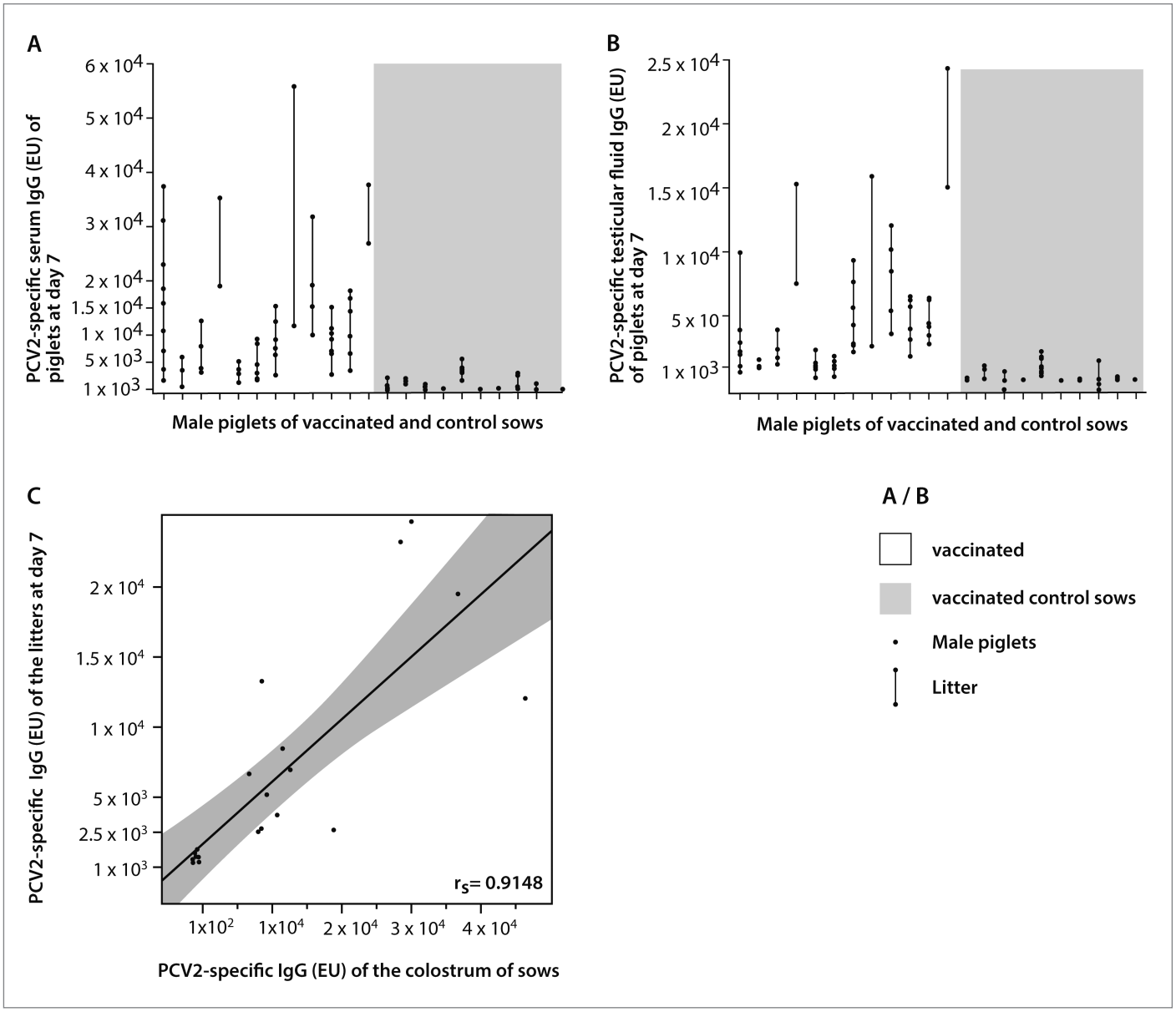

Figure 3: Comparison and correlation of PCV2-specific IgG-antibody titers of male piglets (•) of the same litter (**I**) from vaccinated or control sows. **A**, Antibody titers of male piglets of vaccinated (white) and control sows (grey) from serum and **B**, testicular tissue fluid at castration and **C**, correlation between mean PCV2-specific IgG-antibody titer (in EU) of the litters and colostrum of the sows.

ular tissue fluid was used to differentiate between piglets from vaccinated and unvaccinated sows. This value was predetermined in a ROC-analysis with a sensitivity of 94.92% and a specificity of 79.82%. Testing a single piglet will not render a clear verdict of the PCV2 vaccination status of a herd because of extensive variation among the individual IgG titers of piglets from the same litter. With the help of the binomial distribution, five randomly chosen piglets from different sows with dissimilar parities were tested. If at least four of the five piglets showed a titer > 819 EU in the testicular tissue fluid it was possible to determine the herd of origin to be vaccinated with a reliability of 98%. Conversely, when at least 4 piglets showed a titer < 819 EU, with a confidence of 73% the piglets were born in an unvaccinated herd.

Discussion

IgG can be detected in serum, in lymph and interstitial fluid as well as intracellularly.[14,24] Despite research efforts to replace surgical castration of male piglets in the first week of life, piglet castration remains a worldwide practice to prevent boar taint. In this proof of concept study, we investigated the suitability of using castration

by-products from male progeny for the prediction of PCV2 vaccination status of their mothers and more broadly for herd monitoring.

We observed strong linear correlations between antibody titers from sow serum and colostrum, and between piglet serum and testicular tissue fluid. Antibody titers in piglets are a linear function of the intestinal absorption of colostral antibodies. However, there are large variations among individual piglets within a litter with respect to antibody titers in serum and tissue fluid. The IgG content of sow colostrum varies depending on parity and season, but not among individual teats[9,11] and is not related to litter size.[6] Birth order and birth weight affect essentially the IgG level in the serum of individual piglets.[7] The highest concentrations were measured in piglets with birth order 4 to 6 and the lowest concentrations in piglets born last.[19] The IgG level in piglet serum is adversely affected by inadequate ingestion of colostrum that may occur if the sow has mastitis, as well as with a prolonged interval between birth and suckling and reduced suckling time.[18] Despite the large number of factors affecting IgG content in piglet serum, we observed a good, linear correlation between the IgG concentrations in piglet serum and testicular tissue fluid ($r_s = 0.9269$). The comparison between the median IgG content of piglet serum and testicular tissue fluid revealed that the titers in the tissue fluid were approximately one quarter (23%) of the serum titers.

Maternal antibody titers in the blood of newborn piglets have been shown to decrease by 75% in the first 5 weeks3 and in another study, a decrease of 50% in the same period.[12] This indicates that anti-PCV2 IgG titers in testicular tissue fluid are also affected by the time of castration. Hence, the piglets should always be castrated at the same age if testicular tissue fluid will be used for herd monitoring.

ROC analysis of the data from 22 sows and 111 male piglets revealed a cut-off value for the PCV2 titers in testicular tissue fluid of 819 EU on this farm. This value aids in the differentiation of piglets from vaccinated and unvaccinated sows, but it must be remembered that it is affected by a variety of factors including metritis mastitis agalactia or postpartal dysgalactia syndrome rate in the herd, age of the sows, farrowing management and age of the piglets at castration. Testing of larger numbers of sows and piglets from different herds is required to generate reliable cut-off values. Furthermore, different laboratories are likely to generate different results. They should therefore establish, and continually verify their own cut-off values.

Based on our results, at least five randomly selected piglets from different litters must be tested to allow confirmation with 98% reliability that vaccination of the sows has taken place. If the values of more than one piglet are below the cut-off titer, testing is repeated in five other piglets. If the results remain ambiguous, irregularities in the vaccination protocol should be suspected.

Testing of testicular tissue fluid as used in the present study could also be advantageous for monitoring the success of other vaccinations during pregnancy or to detect deficiencies in vaccination management as well as the passive transfer of colostral immunity. Analysis of tissue fluid of testes from castrated piglets promises to be a powerful tool in pig herd monitoring with a wide range of possible applications. Furthermore, it is a very animal friendly method in which castration waste products can be used to replace painful and time-consuming blood samplings for herd monitoring or to verify vaccination status.

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Pertinence du liquide tissulaire testiculaire des porcelets castrés pour vérifier l'état de vaccination des truies et pour la surveillance du troupeau

Dans une preuve de concept, les anticorps IgG spécifiques au PCV2 provenant de liquide tissulaire testiculaire de porcelets castrés âgés de sept jours ont été mesurés pour vérifier le statut vaccinal de leur mère. Douze truies sélectionnées au hasard ont été vaccinées deux fois au cours du dernier tiers de la gestation avec un vaccin PCV2, tandis que dix témoins n'ont reçu que l'adjuvant. Les titres d'anticorps IgG spécifiques au PCV2 dans le sérum et le colostrum des truies étaient corrélés avec les titres d'anticorps IgG spécifiques au PCV2 dans le sérum et le liquide tissulaire testiculaire de leur progéniture mâle castrée. Les truies vaccinées ont montré des titres moyens d'anticorps IgG spécifiques au PCV2 significativement plus élevés dans le sérum (29250 unités ELISA, UE) et le colostrum (65410 UE) par rapport à respectivement 980 UE et 2630 UE dans le groupe témoin. De plus, des concentrations moyennes d'anticorps significativement plus élevées ont également été mesurées dans le sérum (9362 EU contre 247 EU) et le liquide tissulaire testiculaire (4022 EU contre 354 EU) de porcelets vaccinés par rapport aux porcelets de truies n'ayant reçu que l'adjuvant. Il est important de noter une forte corrélation linéaire entre les anticorps IgG spécifiques au PCV2 dans le sérum des porcelets et ceux présents dans leur liquide tissulaire testiculaire ($r_s = 0{,}9148$). Les titres d'anticorps IgG spécifiques au PCV2 dans le liquide tissulaire testiculaire provenant de cinq porcelets sélectionnés au hasard ont permis de déterminer le statut vaccinal du troupeau avec une fiabilité de 98% pour les truies vaccinées et 73% pour les truies non vaccinées. De plus, l'utilisation de déchets de castration est une méthode très respectueuse des animaux pour remplacer les douloureux et longs prélèvements sanguins pour la surveillance du troupeau ou pour vérifier le statut vaccinal.

Mots-clés: Castration, surveillance, anticorps IgG spécifiques au PCV2, liquide tissulaire testiculaire, statut vaccinal

Verificazione dell'idoneità del liquido tissutale testicolare dei suinetti castrati per controllare lo stato di vaccinazione delle scrofe e per il monitoraggio della mandria

Con una prova di fattibilità, sono stati calcolati gli anticorpi IgG specifici per PCV2 nel liquido testicolare del tessuto testicolare di suinetti di sette giorni castrati, per verificare lo stato di vaccinazione delle scrofe. Dodici scrofe selezionate a caso sono state vaccinate due volte durante l'ultimo trimestre di gestazione con PCV2, mentre dieci hanno ricevuto il solo coadiuvante. I titoli anticorpali specifici PCV2 del siero e del colostro delle scrofe sono stati comparati a quelli del siero e del liquido tissutale testicolare della loro discendenza maschile castrata. Con 29250 unità ELISA (EU) nel siero e 65410 EU nel colostro, le scrofe vaccinate avevano titoli anticorpali IgG specifici per PCV2 significativamente più elevati rispetto ai titoli sierici (980 EU) e del colostro (2630 EU) delle scrofe di controllo. I suinetti provenienti da scrofe vaccinate avevano concentrazioni di anticorpi significativamente più elevate con 9362 EU nel siero e 4022 EU nel liquido tissutale testicolare rispetto ai suinetti provenienti dalle scrofe di controllo trattate con coadiuvanti, che avevano 247 EU nel siero e 354 EU nel liquido tissutale testicolare. È importante notare che è stata trovata una forte correlazione lineare tra gli anticorpi IgG specifici per PCV2 nel siero dei suinetti e nel loro liquido tissutale testicolare ($r_s = 0{,}9148$). I titoli di anticorpi IgG specifici per PCV2 del liquido tissutale testicolare di cinque suinetti selezionati a caso hanno permesso di determinare lo stato di vaccinazione della mandria con un'affidabilità del 98% per le scrofe vaccinate e del 73% per le scrofe non vaccinate. I risultati mostrano che invece di un campionamento del sangue lungo e doloroso, il tessuto tissutale testicolare dei suinetti castrati potrebbe essere utilizzato per il monitoraggio o per determinare lo stato di vaccinazione delle scrofe.

Parole chiave: liquido tissutale testicolare, stato della vaccinazione, castrazione, monitoraggio, anticorpi IgG specifici per PCV2

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